Inhibitors of 15-Lipoxygenase from Orange Peel

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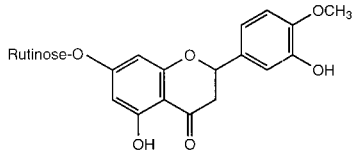
A series of polymethoxylated flavonoids has been isolated from orange peel, and their inhibitory activity toward soybean 15-lipoxygenase was determined. The strongest inhibition was shown by 3,5,6,7,3′,4′-hexamethoxyflavone (IC_{50} = 49 ± 5 μM). Sinensetin, nobiletin, tangeretin, tetramethylscutellarein, and 3,5,6,7,8,3′,4′-heptamethoxyflavone were somewhat less active, with IC_{50} values of 70−86 μM, comparable to the positive control quercetin (IC_{50} = 68 ± 5 μM). Demethylation apparently results in less active compounds, with 5-*O*-demethylsinensetin having an IC_{50} value of 144 ± 10 μM. Some other orange peel constituents were isolated and tested as well, hesperidin (IC_{50} = 180 ± 10 μM) and ferulic acid (111 ± 2 μM), showing moderate activity. The polymethoxylated flavonoids were virtually inactive as scavengers of the diphenylpicrylhydrazyl radical. Hesperidin was only slightly active (24.2 ± 0.7% scavenged at a concentration of 2 mM), and ferulic acid showed good activity (IC_{50} = 86.4 ± 0.7 μM). From this, it appears that orange peel constituents may counteract enzymatic lipid peroxidation processes catalyzed by 15-lipoxygenase in vitro. The radical scavenging activity of orange peel extracts is only modest.

Keywords: *Citrus sinensis; orange peel; flavonoids; 15-lipoxygenase; radical scavengers*

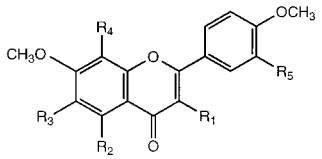
INTRODUCTION

Orange (*Citrus sinensis* Osbeck; Rutaceae) peel is a rich source of flavonoids. Quantitatively, the flavanone hesperidin (**1**) is the most important one. Hesperidin is mainly found in the white (albedo) part of the peel (Walther et al., 1966). It has several reported biological activities. The capillary protective effect of hesperidin has been known for a long time, and the substance is still employed (usually in admixture with other substances, such as the flavone diosmin) in venoprotective pharmaceuticals (Struckmann and Nicolaides, 1994). Other flavanone glycosides (mainly narirutin) are also present, but hesperidin constitutes nearly 90% of the total amount of these compounds (Manthey and Grohmann, 1996; Kawaii et al., 1999).

From a pharmacological point of view, the polymethoxylated flavones found in the flavedo layer of the peel may be of even more interest. More than a dozen such compounds have been reported (Horowitz and Gentili, 1977; Tatum et al., 1978), including substances **2**−**9** shown in Figure 1. Substance **2** has very rarely been reported in *C. sinensis* (Tatum et al., 1978). It is, however, known from other *Citrus* species such as *C. reticulata* (Iinuma et al., 1980; Sugiyama et al., 1993) and *C. tankan* (Cheng et al., 1985). The biological activities of these compounds have been reviewed by Attaway (1994), Middleton and Kandaswami (1994), Benavente-García et al. (1997), and Attaway and Buslig (1998). Antitumor effects (including anticarcinogenic, antimutagenic, antipromotor, antiproliferative, differentiation inducing and antiinvasive properties), anti-inflammatory, anti-aggregatory and anti-allergic activities of polymethoxylated *Citrus* flavonoids have been the subject of numerous studies. Recently, it has been reported that these flavonoids inhibit expression of tumor necrosis factor-α (Manthey et al., 1999). Tangeretin (**6**) may compromise the activity of the anticancer drug tamoxifen (Bracke et al., 1999), so excessive consumption of tangeretin-rich products may be contraindicated in patients during tamoxifen treatment. Several of the methoxylated *Citrus* flavonoids are inhibitors of the P-glycoprotein drug efflux transporter (Takanaga et al., 2000). An anti-ulcer agent containing



1, Hesperidin



Substance	R1	R2	R3	R4	R5
2 (5-Hydroxy-6,7,3′,4′-Tetramethoxyflavone)	H	OH	OCH_3	H	OCH_3
3 (Sinensetin)	H	OCH_3	OCH_3	H	OCH_3
4 (Tetramethylscutellarein)	H	OCH_3	OCH_3	H	H
5 (3,5,7,8,3′,4′-Hexamethoxyflavone)	OCH_3	OCH_3	H	OCH_3	OCH_3
6 (Tangeretin)	H	OCH_3	OCH_3	OCH_3	H
7 (3,5,6,7,3′,4′-Hexamethoxyflavone)	OCH_3	OCH_3	OCH_3	H	OCH_3
8 (Nobiletin)	H	OCH_3	OCH_3	OCH_3	OCH_3
9 (3,5,6,7,8,3′,4′-Heptamethoxyflavone)	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3

Figure 1. Structures of flavonoids isolated from orange peel in this investigation.

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nobiletin (**8**) as the active component has been patented (Hirano, 1994).

The enzyme 15-lipoxygenase (15-LO) has repeatedly been implicated in oxidation of low-density lipoprotein (LDL), a process that is believed to be important for the development of atherosclerosis (Cornicelli and Trivedi, 1999; Steinberg, 1999). Although the involvement of 15-LO has been debated, recent results point toward an anti-atherosclerotic effect in vivo in rabbits for 15-LO inhibitors without anti-oxidant activity (Sendobry et al., 1997; Bocan et al., 1998). Results from experiments in genetically altered mice seem to point in the same direction (Cyrus et al., 1999).

Two of the methoxylated flavonoids found in orange peel, sinensetin (**3**) and tetramethylscutellarein (**4**), which were previously isolated from the medicinal plant *Orthosiphon spicatus*, have been found to inhibit this enzyme (Lyckander and Malterud, 1992). Thus, an investigation of the 15-LO inhibitory properties of the flavonoids from orange peel would seem to be of interest, since orange peel is consumed by humans in, for example, marmalade. To our knowledge, 15-LO inhibition by *Citrus* constituents has not been reported previously. In parallel with this investigation, we studied the radical scavenging activity of orange peel components. The results are reported in this paper.

MATERIALS AND METHODS

Apparatus, Reagents, and Plant Material. NMR spectra (^{1}H and ^{13}C) were recorded at 200 and 50 MHz, respectively, on a Varian Gemini 200 instrument (Varian, Palo Alto, CA). For UV/VIS measurements, a Shimadzu UV-160A instrument equipped with a CPS-240 thermostated cell positioner was employed (Shimadzu, Kyoto, Japan). Centrifugally accelerated TLC was performed on a Chromatotron type 7924T (Harrison Research, Palo Alto, CA). UV irradiation of TLC sheets was carried out with a UVSL 58 dual-wavelength lamp (Ultra-Violet Products, San Gabriel, CA).

Diphenylpicrylhydrazyl (DPPH), 15-lipoxygenase (type 1), and linoleic acid were purchased from Sigma (St. Louis, MO). 5-Hydroxy-6,7,3′,4′-tetramethoxyflavone (**2**), sinensetin (**3**), and tetramethylscutellarein (**4**) were available from previous work (Malterud et al., 1989). Quercetin was from Koch-Light (Colnbrook, England), hesperidin was from Merck (Darmstadt, Germany), and ferulic acid was from Fluka (Buchs, Switzerland). Other chemicals were of the highest purity available.

Oranges were purchased locally and peeled, and the peel was air-dried at room temperature. A sample of the dried peel is deposited in the Department of Pharmacognosy, School of Pharmacy, The University of Oslo.

Extraction and Isolation of Substances. The dried peel was milled to pass through a 4-mm sieve. A 1-kg portion was extracted with 3 × 2.2 L of chloroform for 24 h, and the solvent was removed in vacuo (yield 18.8 g). The chloroform-extracted peel was extracted in the same way with 6 × 2.0 L of methanol (yield 386.1 g).

The chloroform extract was subjected to water vapor distillation, yielding 2.32 g of ethereal oil. The aqueous rest was taken to dryness in vacuo, dissolved in 20 mL of chloroform, and applied to a MPLC column (Büchi, Flawil, Switzerland; 49 × 900 mm) filled with silica gel (40−63 μm; Merck, Darmstadt, Germany). The column was eluted with chloroform containing increasing amounts of ethyl acetate (0.5 L) followed by ethyl acetate (2.5 L), acetone (1.75 L), and methanol (0.75 L). Fractions of 250 mL were collected and combined as indicated by ^{1}H NMR and TLC (Si gel 60 F_{254}, 0.2 mm thickness, Merck) using hexane−ethyl acetate (1:1) or toluene−dioxane−acetic acid (90:25:4) as mobile phases. Spots were visualized by UV irradiation (254 and 366 nm), by spraying with a DPPH solution, or by spraying with $Ce(SO_4)_2$ (1% in 10% aqueous H_2SO_4) followed by heating to 105 °C for 5 min.

The methanol extract was suspended in 1.5 L of water and extracted with 6 × 300 mL of ethyl acetate, yielding an extract (9.16 g), an undissolved part (8.5 g), and an aqueous rest. The ethyl acetate extract was subjected to MPLC as described above, and fractions from the chloroform extract and the ethyl acetate extract, which appeared similar (^{1}H NMR, TLC), were combined.

Further purification was carried out by flash chromatography (Si gel, 40−63 μm, Merck), centrifugally accelerated TLC over 2 mm layers of Si gel F_{254} gipshaltig (Merck), or preparative TLC (Si gel F_{254}, 0.2 mm thickness, Merck). As mobile phases, hexane−ethyl acetate (9:1 or 1:1), toluene−dioxane−acetic acid (180:25:4 or 90:25:4), ethyl acetate, or heptane−2-propanol (6:4) were employed.

The following pure substances were isolated: 5-Hydroxy-6,7,3′,4′-tetramethoxyflavone (**2**), sinensetin (5,6,7,3′,4′-pentamethoxyflavone, **3**), and tetramethylscutellarein (5,6,7,4′-tetramethoxyflavone, **4**) were identified by direct comparison with previously isolated substances (^{1}H NMR, ^{13}C NMR, TLC) (Malterud et al., 1989). 3,5,7,8,3′,4′-Hexamethoxyflavone (**5**), tangeretin (5,6,7,8,4′-pentamethoxyflavone, **6**), 3,5,6,7,3′,4′-hexamethoxyflavone (**7**), nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone, **8**), and 3,5,6,7,8,3′,4′-heptamethoxyflavone (**9**) were identified by comparison of ^{1}H and ^{13}C NMR spectra with literature values (Machida and Osawa, 1989) and by fluorescence color on TLC plates (Tatum and Berry, 1972). In addition, a nonflavonoid substance, ferulic acid, was isolated and identified by comparison of ^{1}H and ^{13}C NMR spectra and TLC mobilities with authentic material. This substance has previously been reported from *C. sinensis* (Stohr and Hermann, 1975).

The insoluble part of the methanol extract after partition between ethyl acetate and water was recrystallized from water, yielding hesperidin (5,3′-dihydroxy-4′-methoxy-7-rutinosyloxyflavanone, **1**), identified by direct comparison (^{1}H and ^{13}C NMR, TLC) with an authentic sample. The aqueous rest from this partition contained mainly carbohydrates (as indicated by ^{1}H NMR) and was not purified further.

Inhibition of 15-LO. Enzyme inhibition was measured as described previously (Lyckander and Malterud, 1992) in borate buffer (0.2 M, pH 9.00) by the increase in absorbance at 234 nm from 30 to 90 s after addition of 15-LO, using linoleic acid (134 μM) as substrate. The final enzyme concentration was 167 U/mL.

Test substances were added as DMSO solutions (final DMSO concentration of 1.6%); DMSO alone was added in control experiments. The enzyme solution was kept on ice, and controls were measured at intervals throughout the experimental periods to ensure that the enzyme activity was constant. All measurements were carried out at least twice, in each instance using six or more parallels of controls and three or more parallels for each concentration of the test substances. Quercetin, a well-known inhibitor of 15-LO (Lyckander and Malterud, 1992), was employed as a positive control.

Calculation of enzyme activity was carried out as previously described (Lyckander and Malterud, 1992), and IC_{50} values were determined by linear interpolation between the measuring points closest to 50% activity. Values are expressed as means ± SD. Student's *t*-test was employed for determination of statistical significance, using a P value of 0.05 or less as a criterion for significant inhibition.

Scavenging of 1,1-Diphenyl-2-picrylhydrazyl (DPPH). To a solution of DPPH in methanol (A_{517} = 1.0; 2.95 mL) was added 50 μL of test substance solution in dimethyl sulfoxide, the mixture was stirred, and the decrease in A_{517} over a 5-min period was measured. Calculation of radical scavenging activity was carried out as previously described (Malterud et al., 1993).

Qualitative Determination of Radical Scavenging on TLC Plates. This was carried out by spraying the TLC plate with a methanolic DPPH solution of a concentration sufficient

to give a violet color to the plate. Radical scavengers were visible as yellow spots (Glavind and Hølmer, 1967).

RESULTS AND DISCUSSION

The flavanone glycoside hesperidin (**1**), the methoxylated flavonoids **2**−**9,** and ferulic acid were isolated from orange peel. All of these substances are previously known from *C. sinensis*. Other methoxylated flavonoids have been reported as trace constituents of orange peel (Horowitz and Gentili, 1977; Tatum et al., 1978). These may presumably correspond to substances giving fluorescent spots observed by us on TLC plates but not isolated in amounts sufficient for identification.

In total, we isolated about 1.0 g of methoxylated flavonoids from 1 kg of orange peel. Of this, nobiletin was the major compound (about 48% of the total amount), followed by sinensetin (24%), tetramethylscutellarein (11%), heptamethoxyflavone (7%), and tangeretin and 3,5,6,7,3′,4′-hexamethoxyflavone (4% each). 5-Hydroxy-6,7,3′,4′-tetramethoxyflavone (0.6%) and 3,5,7,8,3′,4′-hexamethoxyflavone (0.3%) were minor components. Of the latter substance, insufficient amounts for testing of enzyme inhibition were obtained.

These values are based on the weight of isolated compounds and are not analytical values. They are, however, in good accord with previously published values for methoxyflavone content in orange peel (Manthey and Grohmann, 1996) and for distribution of methoxyflavones in orange peel juice (Tatum and Berry, 1972).

In the crude extracts, the highest inhibitory activity toward 15-LO was observed in the chloroform extract (41 ± 3% inhibition at 167 μg/mL). The methanol extract had an inhibitory activity of 9 ± 1% at this concentration. The aqueous phase (after ethyl acetate extraction of the methanol extract suspended in water) was inactive, and the ethereal oil obtained by water vapor distillation was moderately active (12 ± 2%).

The radical scavenging activity was low (although statistically significant; $P < 0.05$) in both the chloroform extract (3.0 ± 0.5%) and the methanol extract (2.1 ± 0.4%), both at 167 μg/mL. The aqueous phase and the ethereal oil were devoid of activity in this assay.

Spraying of TLC plates with DPPH solution revealed two substances active as radical scavengers. These were identified as ferulic acid and hesperidin. The methoxylated flavonoids had low scavenging activity, in accordance with previously published data (Lyckander and Malterud, 1996). Ferulic acid, a known DPPH scavenger (Brand-Williams et al., 1995) had an IC_{50} value in our system of 86.4 ± 0.7 μM. Hesperidin was much weaker (24.4 ± 0.7% scavenged at a concentration of 2 mM). Previous reports (Benavente-García et al., 1997; Suarez et al., 1998) also tend to show only moderate antioxidant and radical scavenging activity for this substance. Thus, while hesperidin has been stated to be the major antioxidant in orange peel (Kroyer, 1986), the presence in lower concentrations of stronger radical scavengers may be of importance for the total scavenging activity. In general, however, orange peel does not seem to be a very good source of radical scavengers, as seen from the low activity of the crude extracts.

Inhibition of enzymatic lipid peroxidation catalyzed by 15-LO was mainly due to the methoxylated flavones. For two of these, sinensetin (**3**) and tetramethylscutellarein (**4**), 15-LO inhibition has been reported previously (Lyckander and Malterud, 1992). To our knowledge, this activity has not been reported for the rest of these compounds. Ferulic acid and hesperidin showed moderate inhibitory activity.

Table 1. Inhibition of 15-Lipoxygenase by Orange Peel Constituents[a]

substance	IC_{50} (μM)
hesperidin (**1**)	180 ± 10
5-hydroxy-6,7,3′,4′-tetramethoxyflavone (**2**)	144 ± 10
sinensetin (**3**)	74 ± 7
tetramethylscutellarein (**4**)	71 ± 7
3,5,7,8,3′,4′-hexamethoxyflavone (**5**)	nt
tangeretin (**6**)	74 ± 5
3,5,6,7,3′,4′-hexamethoxyflavone (**7**)	49 ± 5
nobiletin (**8**)	86 ± 7
3,5,6,7,8,3′,4′-heptamethoxyflavone (**9**)	70 ± 4
ferulic acid	111 ± 2
quercetin (positive control)	68 ± 5

[a] Values are shown ±SD. nt, not tested (due to lack of material).

IC_{50} values for 15-LO inhibition are given in Table 1. 3,5,6,7,3′,4′-Hexamethoxyflavone (**7**) was significantly more active than the other compounds, which were comparable in activity to the positive control quercetin. It appears that the presence of a methoxyl group in position 3 increases inhibitory activity (**7** vs **3**, **9** vs **8**). This effect has not been reported previously. Methoxylation in position 8, however, seems to decrease activity slightly (**8** vs **3**) or to be without effect (**6** vs **4**). Demethylation of the 5-methoxyl group leads to decreased activity (**2** vs **3**). No pronounced difference is observed in 4′-methoxylated substances (**4** and **6**) versus their 3′, 4′-dimethoxylated analogues (**3** and **8**). We have previously shown that for 5,6,7-trioxygenated flavonoids, the B-ring substitution pattern is not important for 15-lipoxygenase inhibition, but for 5,7-dioxygenated ones, ortho dioxygenation in the B ring is required for inhibitory activity (Lyckander and Malterud, 1992).

The 15-LO from soybeans is not identical to the mammalian enzyme. It has, however, repeatedly been shown that there is a good correlation for inhibitory activity toward the two enzymes (Nuhn et al., 1991; Gleason et al., 1995), so soybean 15-LO is commonly used in assays for 15-LO inhibition.

The modulating effects of *Citrus* constituents on arachidonic acid metabolism are not well-known. Nogata et al. (1996) showed that a crude extract of *C. sinensis* (which was not characterized chemically) did not inhibit 12-lipoxygenase, an enzyme different from 15-LO. The extract showed, however, inhibitory activity toward cyclooxygenase. To our knowledge, modulation of 15-LO activity by *Citrus* components has not been reported previously.

Although it is well-known that 15-LO is able to oxidize LDL, the relevance for atherosclerosis development in vivo has been unclear, and the putative role of 15-LO in this respect has been discussed intensively (see, e.g., Feinmark and Cornicelli, 1997; Kühn and Chan, 1997; Cornicelli and Trivedi, 1999; Steinberg, 1999). At present, the role of 15-LO inhibitors as possible antiatherosclerotic agents is still mostly a matter of conjecture, although some recent results with a nonantioxidant inhibitor of 15-LO (Sendobry et al., 1997; Bocan et al., 1998) seem promising.

The methoxylated orange peel flavonoids are nonantioxidant inhibitors of 15-LO as well. However, at present it is not known whether they can counteract atherosclerosis development. This will depend on their bioavailability, metabolism, and their concentration in

the LDL particle. Knowledge about this seems to be mostly lacking, although it has been shown that tangeretin is demethylated and hydroxylated by liver microsomes (Canivenc-Lavier et al., 1993; Nielsen et al., 1998). It is of interest that another lipophilic flavonoid, glabridin, reaches high concentrations in LDL when given orally to mice (Fuhrman et al., 1997).

It has been demonstrated that a combination of ascorbic acid and a *Citrus* extract (from *C. aurantium*) inhibited atherogenesis in hamsters (Vinson et al., 1998). Although hypolipidemic, hypocholesteremic, and antioxidant effects were observed and probably to some extent may explain the observed antiatherosclerotic activity, it would be of interest to elucidate the possible 15-LO inhibitory effect of this combination as well as the content of polymethoxylated flavones in the extract.

Recently, it was reported that short-term supplementation with *Citrus* flavonoids did not influence lipoprotein oxidation in type II diabetic women (Blostein-Fujii et al., 1999). The composition of the supplement was however not given, and it appears uncertain whether it contained any methoxylated flavonoids at all.

Since orange peel is a constituent of marmalade, which is consumed by humans, further research on 15-LO inhibition by orange peel-containing products and on the possible antiatherosclerotic properties of methoxylated *Citrus* flavonoids would seem to be worthwhile.

ABBREVIATIONS USED

15-LO, 15-lipoxygenase; DPPH, 1,1-diphenyl-2-picrylhydrazyl; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MPLC, medium-pressure liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

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